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Genetic characteristics of HIV-1 subtype C envelopes inducing cross-neutralizing antibodies

Cecilia Rademeyer [a], Penny L. Moore [b,*], Natasha Taylor [b], Darren P. Martin [a], Isaac A. Choge [b], Elin S. Gray [b], Haynes W. Sheppard [c], Clive Gray [b], Lynn Morris [b], Carolyn Williamson [a] the HIVNET 028 study team

[a] *Institute of Infectious Diseases and Molecular Medicine, Division of Medical Virology, University of Cape Town, Observatory 7925, Cape Town, South Africa*
[b] *National Institute for Communicable Diseases, Private Bag X4, Sandringham 2131, Johannesburg, South Africa*
[c] *Northern California Center for AIDS Research, UC Davis, Sacramento, California, USA*

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Abstract

This study aimed to characterize genetic features of HIV-1 subtype C envelope glycoproteins capable of eliciting cross-reactive neutralizing antibodies during natural infections. The gp160 sequences were determined for 36 HIV-1 subtype C isolates (donor viruses) from infected individuals residing in Malawi, Zimbabwe, Zambia and South Africa, whose sera displayed a range of cross-neutralizing activities against a panel of 5 subtype C and 5 subtype B viruses (panel viruses). Hierarchical clustering analysis of neutralization data of the panel viruses predicted phylogenetic relationships between subtype B and C panel viruses, suggesting some subtype-specific neutralization determinants. A similar comparison of subtype C donor viruses showed no significant correlation; however of three donor sequence pairs resolvable by phylogenetic analysis, two were also associated within the neutralization clustering dendrogram, suggesting that closely related viruses may elicit antibodies targeting common neutralization determinants. Significantly, viruses that had shorter V1–V4 loops induced antibodies that showed more neutralization breadth against the subtype C panel viruses ($p=0.0135$). This study indicates that that some structural features of envelope, such as shorter variable loops, may facilitate the elicitation of cross-reactive neutralizing antibodies in natural infections. Collectively these data provide some insights into design features of an envelope immunogen aimed at inducing neutralizing antibodies.

Keywords: HIV-1 subtype C; Neutralizing antibodies; Envelope

Introduction

The HIV-1 epidemic has continued to expand with an estimated 39.5 million people worldwide now infected (UNAIDS/WHO, 2006) making a preventative vaccine an urgent global health priority. It is widely believed that such a vaccine would require the induction of both virus-specific CD8+ T cells and neutralizing antibodies (nAbs) (Letvin et al., 2002; Mascola, 2003). Evidence for the role of nAbs comes from studies with non-human primates which have confirmed the protective capacity of passively transferred nAbs (Baba et al., 2000; Hofmann-Lehmann et al., 2001; Mascola et al., 1999, 2000; Parren et al., 2001; Veazey et al., 2003) and from recent data showing that, in some HIV-infected individuals, monoclonal nAbs can reduce the rate of viral rebound following a structured treatment interruption (Trkola et al., 2005).

Despite considerable effort, there has been little progress in creating a vaccine capable of eliciting nAbs, most likely due to difficulties in designing an immunogen that sufficiently resembles the native Env trimer (Garber et al., 2004). Immunogen design is hampered by Env variable loops which sterically shield critical epitopes. In addition, an "evolving glycan shield" and the occlusion of neutralizing antibody sites by oligomerization present further challenges (Garber et al., 2004; Wei et al., 2003). High envelope sequence diversity may also diminish the ability of a vaccine to induce broadly cross-reactive antibodies.

* Corresponding author. Fax: +2711 386 6453.
E-mail address: pennym@nicd.ac.za (P.L. Moore).

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Although Bures and coworkers (Bures et al., 2002) demonstrated regional serotypes, with sera from South Africa preferentially neutralizing subtype C isolates from South Africa compared to those from Malawi, there are extensive data which demonstrate cross-neutralization between subtypes. This suggests that there may be neutralization determinants common to different HIV-1 subtypes (Kostrikis et al., 1996; Moore et al., 1996; Nyambi et al., 1996; Weber et al., 1996).

To design an effective Env subunit vaccine, further data are needed to determine which portions of Env are most useful for inclusion in a vaccine immunogen. While there are many studies investigating the neutralization properties of serum (Gray et al., 2007; Li et al., 2006a; Richman et al., 2003; Wei et al., 2003) and the identification of neutralization epitopes (Binley et al., 2004; Gray et al., 2006; Zwick et al., 2005), only a few studies have investigated the properties of viruses in natural infections which are associated with the induction of these antibodies (Li et al., 2006b). To identify the genetic characteristics of HIV-1 subtype C envelopes capable of eliciting broadly cross-reactive nAbs, we analyzed gp160 sequences from 36 HIV-1 subtype C infected individuals for whom neutralization data from matched serum samples were available. Our data indicate that there is a limited correlation between the phylogenetic relatedness of viruses and the cross-reactivity of the nAbs they induce. Also we show that some structural features of envelope, such as shorter variable loops, may facilitate the elicitation of cross-reactive nAbs in natural infections.

Results

The HIVNET 028 study is a natural history study that was conducted to evaluate immune responses to HIV-1 subtype C infection (Gray et al., 2005). Serum from individuals residing in Malawi, Zambia, Zimbabwe and South Africa was previously used to examine nAb responses against a panel of five subtype C and five subtype B viruses (referred to hereafter as panel viruses) (Taylor et al., paper in preparation). There was extensive cross-neutralization of the subtype B and C panel viruses by subtype C sera suggesting common neutralization epitopes between subtypes: the average percentage of sera neutralizing each of the subtype C panel isolates was 56% compared to an average of 57% for the subtype B panel isolates. The gp160 region from 36 of these individuals was sequenced (referred to hereafter as donor viruses) using plasma samples collected at the same time point as serum samples used in the neutralization assays. All donor virus nucleotide sequences grouped with reference subtype C sequences with no evidence of inter-subtype recombination (results not shown). A maximum likelihood phylogenetic tree constructed using amino acid sequence data from donor viruses indicated potentially significant clustering (an excess of 50% bootstrap support) of only three sequence pairs (Fig. 1A). Although all three pairs were sampled from the same geographical region, in general there was no clustering of subtype C viruses based on their geographical origin. The subtype B and C panel viruses clustered separately as expected (Fig. 1B).

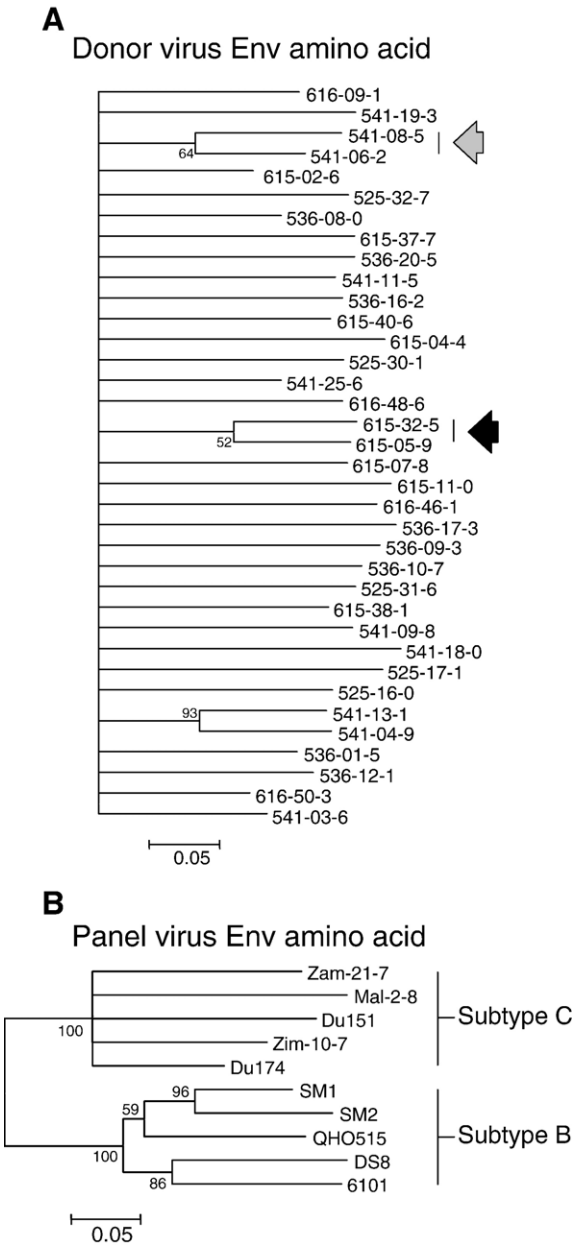

Fig. 1. Maximum likelihood phylogenetic trees indicating the Env amino acid sequence relationships between: (A) donor viruses; (B) panel viruses. Numbers associated with branches represent the percentage of 100 nonparametric bootstrap datasets supporting their existence. Branches with less than 50% support have been collapsed to clarify the mostly unresolved star-like nature of the subtype C virus phylogenies. Arrows indicate well supported relationships between sequence pairs that also cluster closely together in the neutralization dendrogram.

Genetic properties of panel viruses

Since variable loop lengths and predicted *N*-glycosylation (PNG) site numbers may impact neutralization sensitivity, we compared these features of the 5 subtype B and 5 subtype C panel virus sequences. There were no significant differences between variable loop lengths and PNG numbers in the subtype B and C panel virus sequences. However, the subtype C Env proteins tended to have slightly shorter variable loops (median

V1–V2=63 aa and V1–V4=277 aa) and fewer predicted glycosylation sites (median=29) compared to the subtype B proteins (median V1–V2=67 aa, V1–V4=282 aa, and PNG=31).

We then looked at the relationship between variable loop length, PNG number and neutralization sensitivity. There was a significant inverse correlation between V1–V2 length in the subtype C panel viruses and the median titer at which they were neutralized (Spearman $r=-0.7723$, $p=0.0126$). There were, however, no significant correlations between neutralization sensitivity and either V1–V4 length or PNG number for both subtypes. Since the subtype C viruses were not more sensitive to neutralization than the subtype B panel viruses, the impact of the shorter V1–V2 loop on the overall neutralization phenotype of subtype C viruses is probably minimal.

Relationship between donor sequences and neutralized panel viruses

We investigated whether the panel viruses were more easily neutralized by antibodies elicited by viruses to which they were genetically more closely related. To do this, the amino acid pair-wise distances of the panel viruses were compared to those of donor viruses which either did, or did not, induce a neutralizing response. The overall median percentage amino acid pair-wise distance between panel and donor virus sequences from neutralizing sera was 16.4% ($n=183$) compared to 15.5% ($n=138$) for sequences from sera that were unable to neutralize panel viruses. The difference in percentage pair-wise distance was higher for the subtype B panel [23% ($n=85$) for neutralizing sera; 24% ($n=59$) for non-neutralizing sera] compared to the subtype C panel [14% for both neutralizing ($n=98$) and non-neutralizing ($n=79$) sera] as expected given that all donor viruses were subtype C (Fig. 2). Donor viruses eliciting a neutralizing response to three panel viruses (Mal-2-8, Du174 and QH0515) were more closely related to these panel viruses than were donor viruses that did not induce a neutralizing response. However, the opposite was found for six other panel viruses. None of these differences was statistically significant. Furthermore, no correlation was observed between the amino acid pair-wise distance between panel and donor virus sequences (for subtypes B and C separately) and the neutralization titers. These data suggest that antibodies elicited by donor viruses are not obviously more likely to neutralize viruses with which they share the greatest genetic similarity, as measured by amino acid pair-wise distances.

Hierarchical neutralization clustering

We used hierarchical neutralization clustering to classify the capacity of donor sera to neutralize panel viruses (Fig. 3). Donor sera with the most potent neutralizing activity are grouped in the top half of the neutralization tree while sera with the least potent activity are grouped in the bottom half of the tree. Similarly, panel viruses that are most potently neutralized cluster on the left

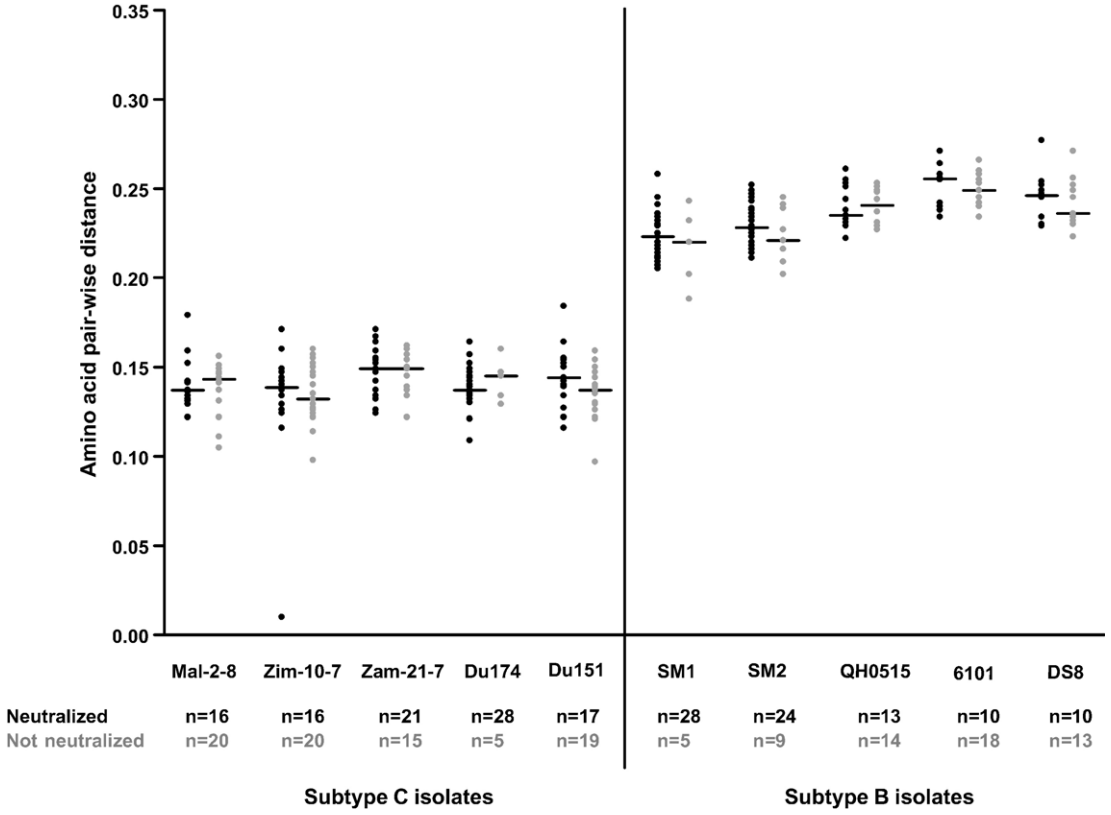

Fig. 2. Genetic relationship between subtype C and B panel viruses and subtype C sequences from neutralizing sera (black circles) and non-neutralizing sera (gray circles) with median amino acid pair-wise distance indicated by a horizontal line. Panel isolates are indicated along the *x*-axis and amino acid pair-wise distances along the *y*-axis.

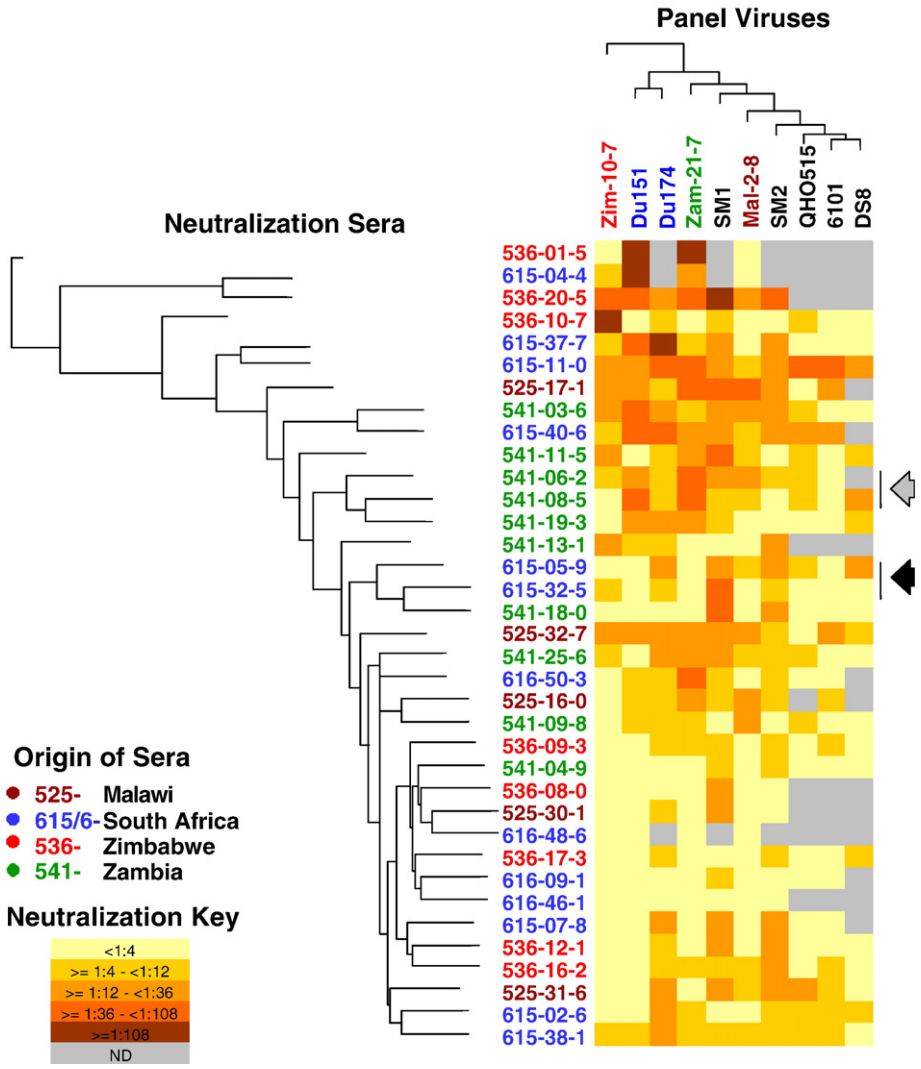

Fig. 3. Hierarchical clustering of 36 donor sera and ten panel viruses according to ID_{80} neutralization potencies and susceptibilities, respectively. The dendrogram on the left was constructed by clustering sera according to their neutralization capacity. Similarly, the dendrogram at the top was constructed by clustering panel viruses according to their neutralization susceptibilities. This resulting two-dimensional clustering pattern was color-coded in order to clarify neutralization profiles. Arrows indicate closely clustered sera that were induced by viruses that were significantly clustered by phylogenetic analysis of the gp160 amino acid sequence. Legends indicate geographic origins and neutralization potencies. ND=not done.

of the panel virus neutralization tree while those viruses least potently neutralized cluster on the right of the tree.

We compared the topologies of the donor and panel virus neutralization dendrograms with their corresponding gp160 amino acid sequences phylogenetic trees and found good evidence that the panel virus neutralization tree (Fig. 3) more closely resembles the phylogenetic tree (Fig. 1B) than would have been expected by chance ($p=0.034$ using the permutation test described in Materials and methods). This suggests that the panel viruses contain some neutralization determinants that are subtype-specific.

There was, however, no significant correspondence between the neutralization clustering and phylogenetic relationships of donor viruses ($p=0.584$). Despite this it is noteworthy that of three sequence pairs grouping together in the mostly star-like donor virus gp160 tree, two of these pairs also clustered closely in the neutralization tree (black and gray arrows in Figs. 1A and 3). It is possible, therefore, that the gp160 sequences of these two pairs of viruses have common genetically determined immunogenic features.

There are three likely reasons why we observed concordance between the neutralization clustering and phylogenetic trees of panel viruses but find no concordance between the trees for the donor viruses: (1) the panel viruses are more diverse and yield a phylogenetic tree which distinguishes between subtype B and C and resolves some clusters (with bootstrap values >80%) within subtype B, whereas all donor viruses are subtype C and these yield a very poorly resolved phylogenetic tree; (2) the neutralization clustering dendrogram for the panel viruses is based on up to 36 informative characters whereas the dendrogram for the donor viruses is based on at most ten informative characters; (3) the ongoing evolution of plasma virus means that the contemporaneous virus (sequenced here) may differ from the virus which elicited the nAbs.

Although there will be considerably less envelope variation within an individual over this period of time compared to the extent of variation between individuals, the relationship between donor serum neutralization and the phylogenetic tree of the donor virus may be somewhat weakened as a result. Given more panel viruses and a more diverse set of donor viruses, it is possible that concordance between donor virus neutralization and phylogenetic trees could be detectable.

Relationship of donor virus loop length and number of PNGS with neutralizing antibody responses

We investigated whether the length of the V1–V2 and V1–V4 regions of donor viruses affected the antibody responses they elicited. A comparison of V1–V2 loop lengths of donor viruses that induced nAbs, with those of donor viruses that did not induce nAbs, indicated that eight of the ten panel viruses were neutralized more efficiently with sera induced by donor viruses that had shorter loop lengths. This trend was, however, not statistically significant when each panel virus was analyzed separately (Fig. 4A). However, when donor virus sequences were analyzed collectively, the V1–V2 lengths of viruses inducing neutralizing sera against subtype C panel viruses were significantly shorter (median, 65.5 amino acids) than those from donor viruses inducing non-neutralizing sera (median, 69 amino acids, $p=0.0296$; Fig. 4A). While there was a similar trend for shorter V1–V2 sequences of donor viruses to induce more potently neutralizing sera against subtype B panel viruses, this was not significant ($p=0.6593$).

A similar trend was observed when the entire V1–V4 region was analyzed. It was found that neutralization of eight of the ten panel viruses was better mediated by sera induced by donor viruses with shorter V1–V4 regions (Fig. 4B). This trend was significant for two panel viruses [Mal-2-8 ($p=0.04$) and QH0515 ($p=0.0153$)]. When subtype C data were analyzed collectively, the V1–V4 amino lengths of donor viruses inducing neutralizing sera were also significantly shorter (median, 276 amino acids) than those from donor viruses that failed to induce neutralizing sera (median, 282 amino acids, $p=0.0135$). Similar to the V1–V2 analysis, there was no statistically significant association between V1–V4 length and the ability of donor viruses to induce neutralizing sera against subtype B panel viruses ($p=0.4164$).

We also examined the relationship between the number of PNGs in the donor viruses and the neutralization capacity of the sera they induced. The number of PNGs per sequence was analyzed with respect to neutralization capacity for each serum versus the panel viruses. With the exception of sera which neutralized panel virus Mal-2-8, and independent of the panel virus subtype, no significant differences were observed in the number of PNGs in donor virus sequences that induced sera which neutralized panel viruses versus those donor viruses that failed to induce neutralizing sera. This was true whether we analyzed either the number of PNGs over the entire gp160 region or only those within the V1–V4 or V1–V2 regions. Donor virus sequences inducing sera that neutralized panel virus Mal-2-8 had significantly reduced PNG numbers relative to those predicted in donor sequences that did not induce sera that neutralized Mal-2-8 ($p=0.0444$ over the entire gp160; $p=0.0349$ over the V1–V4 region alone).

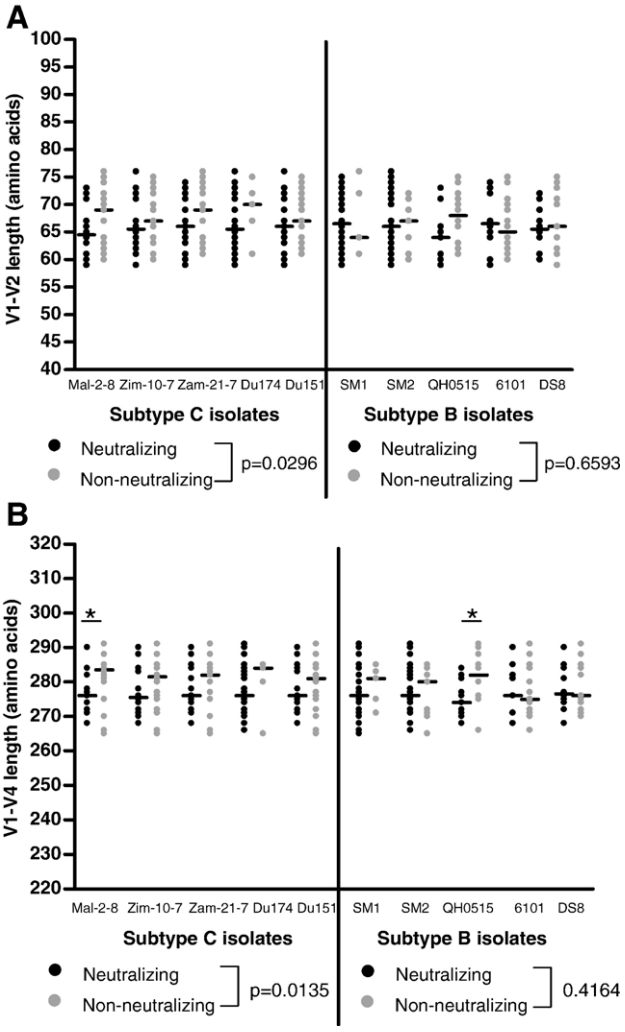

Fig. 4. (A) V1–V2 or (B) V1–V4 amino acid length of donor sequences analyzed according to whether the corresponding serum sample neutralized (black circles) or did not neutralize (gray circles) each of the panel viruses. The median length is indicated by a horizontal line. Collective statistical analysis for subtype C and B isolates is shown below the *x*-axis. Asterisk indicates $p<0.05$.

Discussion

This study aimed to characterize genetic features of HIV-1 subtype C envelope sequences capable of eliciting cross-reactive nAbs in natural infection. Sequences were analyzed from 36 individuals (from Malawi, Zimbabwe, Zambia and South Africa) whose sera displayed a range of cross-neutralizing abilities against a panel of five subtype C and five subtype B viruses. An important phylogenetic feature captured in the hierarchical neutralization clustering dendrogram was the division of subtype C and B panel viruses which provided evidence that neutralization potency has some degree of subtype specificity. Also, two pairs of closely related donor viruses were identified in the phylogenetic tree that also clus-

tered closely in the neutralization dendrogram further suggesting that closely related viruses in different people may elicit antibodies targeting common neutralization determinants. Finally, we provide evidence that viruses with shorter variable loops in natural infections are more likely to induce cross-reactive antibodies. This relationship was observed for neutralization of subtype C but not subtype B panel viruses, further supporting the existence of subtype-specific differences in neutralization sensitivity.

The HIV-1 subtype C infected patients in this study, estimated to have been infected for a median period of 17 months (range 10 to 25 months), showed a broad range of abilities to cross-neutralize both subtypes B and C panel viruses. However, a significant inverse correlation was observed between the median neutralization titer and the length of the V1–V2 region of the subtype C panel viruses. This is similar to what has been reported previously by Li et al. (2006a) who demonstrated an inverse correlation between the length of the V1–V4 region and neutralization susceptibility in HIV-1 subtype C infection. These data contribute to a considerable body of evidence implicating variable loops in (1) occlusion of antibody sensitive regions of the viral envelope from monoclonal and plasma antibodies (Cao et al., 1997; Cheng-Mayer et al., 1999; Ly and Stamatatos, 2000; Pinter et al., 2004; Sagar et al., 2006), (2) neutralization escape of subtype A and B viruses *in vivo* (Cao et al., 1997; Cheng-Mayer et al., 1999; Ly and Stamatatos, 2000; Pinter et al., 2004; Sagar et al., 2006; Wei et al., 2003), and (3) moderation of virus neutralization sensitivity in subtypes A and C (Derdeyn et al., 2004; Gray et al., 2007; Li et al., 2006a; Sagar et al., 2006). More data on the antigenic targets of nAbs and the structural features of HIV-1 envelopes from different genetic subtypes are needed in order to clarify some of these findings.

It is well known that serum is more likely to neutralize autologous virus from earlier time points (Gray et al., 2007; Richman et al., 2003; Wei et al., 2003). However, viruses that were genetically more similar to a panel virus were no more likely to elicit antibodies able to neutralize it than viruses more distantly related to it. This is not surprising given that this method looks at primary Env sequences whereas epitopes are influenced by both the tertiary structure of the envelope and the quaternary nature of the trimer. To investigate this in more depth we compared the topology of neutralization clustering and phylogenetic trees constructed from the donor and panel virus gp160 amino acid sequences. Although the donor virus phylogenetic tree was highly unresolved, two pairs of sequences clustered in both this and the neutralization tree, implying that viruses with similar sequences might elicit similar nAbs in different people. To explore concordance between phylogenetic and neutralization clustering trees further, data would be needed for both a more phylogenetically structured set of donor virus sequences and a greater number of panel viruses. That such a concordance may exist is evidenced by the fact that we have found a definite association between panel virus genotype and neutralization phenotype. With only one exception the neutralization tree showed a reasonably clear split between panel viruses of different subtypes, indicating that at least a fraction of neutralization determinants are both specific to, and fairly common within, subtypes. Our data therefore add further support to previous claims that South African subtype C viruses have shared neutralization determinants (Bures et al., 2002). Here we have extended that report to include subtype C viruses circulating in Zambia and Zimbabwe. We suggest, therefore, that some benefit may be derived from producing subtype C specific Env based vaccines but also that the magnitude of this benefit would probably not be overwhelming in that there is, for example, clear evidence of cross-neutralization of subtype C and B viruses in 28 of the 36 sera we have tested.

In vaccine studies, the Env variable loop lengths have been implicated in determining the degree to which nAbs are elicited (Srivastava et al., 2003). It has been proposed that Env proteins with comparatively short variable loops might be responsible for autologous neutralizing antibody titers in subtype C infected individuals that are approximately 3.5-fold higher than those seen in individuals infected with subtype B viruses which generally express Env proteins with less compact loops (Li et al., 2006a). We found that there was a relationship between the neutralization capacity of sera and the variable loop lengths of the viruses inducing the sera. Comparison of both the V1–V2 and V1–V4 lengths of donor viruses from neutralizing sera with those from non-neutralizing sera showed that, in general, sera which neutralized a particular panel virus had, on average, shorter V1–V2 and V1–V4 regions than did viruses from sera unable to neutralize the panel virus. Whereas this trend was statistically significant for both the V1–V2 and V1–V4 lengths when the subtype C panel viruses were analyzed collectively, it was not significant for the subtype B panel viruses, which suggests that there may be some inter-subtype differences in neutralization determinants. These data suggest that polyclonal sera elicited during natural infections in response to viruses with shorter V1–V2 (and shorter V1–V4 region) lengths were in general more effective at neutralizing our panel viruses. This trend was independent of the variable loop (V1–V2 or V1–V4) lengths of the individual panel viruses (data not shown).

Importantly, we could detect no significant influence of the number of predicted PNGs in donor sequences on their capacities to induce neutralizing sera. Reduced glycosylation of the infecting virus in this study is therefore not linked to elicitation of cross-reactive nAbs as has been suggested (Pantophlet and Burton, 2006).

In conclusion, we found evidence for both an association between the neutralization characteristics of viruses and their phylogenetic relatedness, and the existence of subtype-specific genetic neutralization determinants. However, sequences of donor viruses associated with neutralizing sera were on the whole not characterized by lower amino acid sequence identity distances compared with sequences from non-neutralizing sera. There was, however, evidence that viruses with shorter V1–V2 loops (and shorter V1–V4 regions) elicited antibodies that were capable of more effectively neutralizing panel isolates. This effect has been implied in other studies, but this is the first study to demonstrate it in natural infections. These data therefore provide further insights into necessary design features of an Env based vaccine immunogen that is capable of effectively eliciting broadly protective anti-HIV neutralizing antibody responses.

Materials and methods

Plasma and serum samples

Serum samples used in neutralization assays, and blood plasma samples used to determine Env sequences, were collected as part of the HIVNET 028 study from 36 HIV-1 positive individuals originating from four countries: Malawi (MW, 525-prefix, $n=5$), Zimbabwe (ZW, 536-prefix, $n=8$), Zambia (ZM, 541-prefix, $n=10$), and South Africa (ZA,; 615-prefix for Durban samples, $n=9$; 616-prefix for Johannesburg samples, $n=4$) (Table 1). Most individuals (89%) were females and the group had a median age of 28. Individuals in this study had median plasma viral loads of 1.6×10^4 genome copies ml^{-1} and correspondingly had relatively low CD4+ T cell counts (median of 180 cells/μl). Date of infection was defined as the midpoint between the last antibody negative ELISA date and the first antibody positive ELISA. The median time from infection was 17 months (range 10 to 25 months). Patient demographic data and clinical information were generated as part of the HIVNET 028 study by co-investigators and are described in Gray et al. (2005). Ethical approval for all samples was obtained from institutional review boards of the University of the Witwatersrand and the University of Cape Town, South Africa.

RT-PCR from blood plasma

RNA was extracted from blood plasma and cDNA of the HIV-1 *env*gp160 was generated through reverse transcription (Invitrogen Thermoscript™ RT-PCR System; GmbH, Karlsruhe, Germany). This was followed by a nested PCR reaction using first-round primers G-f (5′-AAGCGGAGACAGCGACGAAG-3′; HXB2 position 5981–6000) and *env*L-r (5′-AAGGCACCTGAGGTCTGACTGG-3′; HXB2 position 9001–9022) using Expand Long template PCR System (Roche Molecular Biochemicals). Cycling conditions were as follows: 1 cycle of 94 °C for 2 min; 10 cycles of 94 °C for 10 s; 50 °C for 30 s and 68 °C for 2 min; 25 cycles of 94 °C for 15 s; 50 °C for 30 s; 68 °C

Table 1
Clinical information for 36 HIV-1 positive donors

Origin	Sample identifier	Estimated months post-infection [a]	Age	Gender	CD4 count (cells/μl)	Viral load (RNA copies/ml)
Malawi	525-00016-0	15.0	44	M	340	53140
	525-00017-1	16.3	28	M	253	68521
	525-00030-1	NA	18	F	608	8583
	525-00031-6	NA	36	F	460	5014
	525-00032-7	12.2	30	F	239	67003
Zimbabwe	536-00001-5	18.8	24	F	193	25079
	536-00008-0	12.0	18–45	F	455	20439
	536-00009-3	9.8	29	F	528	201238
	536-00010-7	14.9	18–45	F	324	2936
	536-00012-1	NA	27	F	610	15588
	536-00016-2	NA	18–45	F	424	4160
	536-00017-3	10.7	28	F	167	46328
	536-00020-5	13.7	20	F	780	6189
Zambia	541-00003-6	25.2	NA	F	149	15556
	541-00004-9	24.1	29	F	453	37706
	541-00006-2	22.6	18–45	F	250	3880
	541-00008-5	24.0	18–45	F	136	115968
	541-00009-8	24.6	18–45	M	318	5668
	541-00011-5	24.1	18–45	F	360	26329
	541-00013-1	22.8	22	F	326	9533
	541-00018-0	22.7	30	F	216	25249
	541-00019-3	21.5	18–45	F	476	93697
	541-00025-6	24.2	33	M	256	14089
Durban, South Africa	615-00002-6	12.9	18–45	F	NA	46984
	615-00004-4	15.8	36	F	522	14199
	615-00005-9	17.8	18–45	F	600	6841
	615-00007-8	21.1	18–45	F	345	5726
	615-00011-0	18.6	25	F	448	8048
	615-00032-5	14.1	NA	F	255	16952
	615-00037-7	13.6	NA	F	32	177259
	615-00038-1	13.6	44	F	592	2349
	615-00040-6	13.7	28	F	180	385188
Johannesburg, South Africa	616-00009-1	NA	NA	F	371	15565
	616-00046-1	NA	NA	NA	45	202735
	616-00048-6	NA	NA	F	538	NA
	616-00050-3	NA	NA	F	384	9639

NA, not available.
[a] Estimated date of infection defined as midpoint between last antibody negative and first antibody positive ELISA.

for 2 min plus a 20 s increase for each cycle at this step. The final cycle had a 68 °C extension step for 7 min. Second round reactions were performed using *env*Ff (5′-TCATAGCAATAGTTGTGTGG-3′; position 6111–6130) and *env*Kr (5′-CTTATAGCAGGCCATCC-3′; position 8830–8846) generating a 2736 bp *env*FK fragment. Reactions (50 μl) used ExSel High Fidelity DNA Polymerase (JMR Holdings, Sevenoaks, UK) with 1× reaction buffer, 0.2 mM of each dNTP, 5 pmol of each primer and 0.625U ExSel taq. PCR cycling conditions used for this second round were identical except that annealing temperature was increased to 55 °C.

Where amplification of this fragment failed, five smaller overlapping fragments were generated using primers EnvAf (5′-GAAAGAGCAGAAGACAGTGGC-3′; position 6203–6223) and *env*Er (5′-TTAGAATCGCATAACCAG-3′; position 6886–6903) for an *env*AE fragment (702 bp); *env*Bf (5′-TAACACAAGCCTGTCCAAAGGT-3′; position 6826–6847) and *env*Br (5′-AATTTCTAGGTCCCCTCCTGA-3′; position 7317–7337) for an *env*B fragment (512 bp); *env*Df (5′-AGCACATTGTAACATTAGT-3′; position 7208–7226) and *env*Ar (5′-TGCTGCTCCCAAGAACCCAA-3′; position 7783–7802) for an *env*DA fragment (594 bp); *env*Mf (5′-GAGGAGATATGAGGGACAATTGG-3′; position 7638–7661) and *env*Nr (5′-GGTGAGTATCCCTGCCTAACTCTA-3′; position 8342–8365) for an *env*MN fragment (727 bp); *env*Nf (5′-TGACCTGGATGCAGTGG-3′; position 8101–8117) and *env*Kr (5′-CTTATAGCAGGCCATCC-3′; position 8846–8830) for an *env*NK fragment (745 bp). These 50 μl reactions were performed using Supertherm DNA polymerase (JMR Holdings, Sevenoaks, UK) with 1× reaction buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 5 pmol of each primer and 0.625 U Supertherm DNA polymerase. PCR cycling conditions used for these fragments were 1 cycle of 94 °C for 2 min, 30 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s with a final extension step at 72 °C for 7 min.

Sequencing and sequence analysis

DNA amplicons were purified by QIAquick PCR Purification Kit (Qiagen, Valencia CA, USA). Both strands were sequenced directly with five pairs of second-round overlapping primers using the ABI PRISM® BigDye™ terminator V3.0 Cycle sequencing Ready reaction Kit and an ABI PRISM™ 3100 Genetic Analyser (Applied Biosystems, Warrington, UK). Nucleotide sequences were edited using ChromasPro V.1.11 (www.technelysium.com.au/ChromasPro.html) and aligned and translated using BioEdit version 5.0.2 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) (Hall, 1999). Phylogenetic relatedness of sequences was inferred by maximum likelihood (JTT model, 100 full maximum likelihood nonparametric bootstrap replicates) using PhyML (Guindon and Gascuel, 2003).

Amino acid distance analysis was performed with uncorrected (Hamming) distances calculated in MEGA version 2.1. Percentage nucleotide diversity was determined using the Kimura 2-parameter model. The number of potential N-linked glycosylation (PNG) sites was determined using the program N-glycosite (http://www.hiv.lanl.gov/content/hiv-db/GLYCOSITE/glycosite.html).

Neutralization assays

Serum samples from 36 individuals were tested in PBMC neutralization assays against a panel of 10 primary viruses. This included Du151, the South African subtype C prototype vaccine strain (isolated 2 months after infection), and Du174 (isolated 14 months after infection) both from Durban, South Africa (Williamson et al., 2003); a Malawian isolate (Mal-2-8); a Zimbabwean isolate (Zim-10-7); and a Zambian isolate (Zam-21-7). These latter 3 subtype C viruses were isolated from stored HIVNET 028 PBMC collected within 6 months of infection, except for the Zambian isolate where exact date of infection was not known. The remaining five viruses were subtype B isolates: one obtained within 2 months of infection from a male residing in the USA (6101); one from an male living in Trinidad who was estimated to have been infected 1 month earlier (QH0515) (Li et al., 2005); two from individuals in South Africa undergoing seroconversion illness (SM1 and SM2); and one from a South African AIDS patient, DS8. Subtype C viruses and one of the subtype B viruses (QH0515) were from heterosexual transmissions and the subtype B viruses were from homosexual transmissions. All HIV-1 isolates utilized the CCR5 co-receptor (NSI phenotype), except for DS8 which used CXCR4 (SI phenotype). A total of 177 neutralization assays were done using the 5 subtype C viruses (3 not done due to limited serum quantities) and 144 assays (37 not done) using the subtype B panel viruses. Neutralization titers were calculated as the reciprocal of the serum dilution that reduced p24 antigen production by 80% relative to a negative control serum sample from an HIV-negative donor (ID_{80}) (Taylor et al., paper in preparation).

Hierarchical neutralization clustering

Dendrograms grouping sera and viruses based on the similarity of either their neutralization susceptibility (for panel viruses) or their ability to neutralize panel viruses (for donor viruses) were generated using the Mcquitty agglomeration method implemented in R (The R Foundation for Statistical Computation version 2.0.1, 2004) (Ihaka and Gentleman, 1996). This method has previously been applied by Binley et al. (2004) to classify viruses into immunotypes. Actual ID_{80} neutralization titers were used in generating dendrograms. Neutralization potency was visualized by categorizing neutralization titers into five color-coded groups: (1) samples with no detectable neutralization (titers of <1:4), (2) samples with titers of >1:4 to <1:12, (3) samples with titers of >1:12 to <1:36, (4) samples with titers of >1:36 to <1:108 and (5) samples with titers of >1:108 indicating highly effective neutralization.

Statistical analyses

Nonparametric regression analyses (Spearman's Rank) were carried out using GraphPad Prism 4.0. Correlations were considered statistically significant when p values were smaller than or equal to 0.05. Evidence of correlation between neutralization clustering and phylogenetic relatedness of Env amino acid sequences was examined using a permutation test. The

maximum likelihood tree of the Env amino acid sequences and an enforced topology corresponding to that of the inferred neutralization clustering dendrogram was determined using PhyML (JTT model; Guindon and Gascuel, 2003). The likelihood of this tree was then compared to those of 1000 trees constructed using datasets in which the identities of Env sequences had been randomly shuffled. The probability of the neutralization clustering dendrogram topology containing significantly more phylogenetic information than could be accounted for by chance corresponded to the proportion of randomized datasets that yielded a higher likelihood estimate than the actual dataset.

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